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IN THE U.S. PATENT & TRADEMARK OFFICE

Applicants: Yukoh HIEI et al

Serial No.: 08/668,464 Group: 1649

Filed: June 25, 1996 Examiner: Benzion

For: METHOD FOR TRANSFORMING MONOCOTYLEDONS

DECLARATION UNDER 37 C.F.R. §1.132

Honorable Commissioner of Patents and Trademarks

Washington, D.C., 20231

Sir:

I, Yukoh HIEI, a nation of Japan, residing at c/o Japan Tobacco Inc., Plant Breeding and Genetics Research Laboratory, 700, Higashibara, Toyoda-cho, Iwata-gun, Shizuoka 438, Japan, do hereby declare as follows:

I am a co-applicant of the invention as described and claimed in the specification of the above-identified application.

I am familiar with the Final Office Action dated February 13, 1998, in which claims 1 - 16 are rejected.

To show the patentability of the present invention, I carried out the experiments described below.

Materials and Methods

1. Investigation of Expression of Introduced Genes in Progenies of Transformants

Seeds of self-pollinated progenies (R1 generation) of the 3 transformants (line numbers 1-3) among the transformants shown in Table 12 on page 56 of the specification of the present application, and 7

transformants (line numbers 4-10) among the transformants shown in Table 13 on page 56 of the specification of the present application were sterilized with 70% ethanol and 1% sodium hypochlorite and were placed on 1/2N6 medium (page 19, lines 7-10 of the specification of the present application). From the seedlings after 7 days from the beginning of the culturing, pieces of leaves were sampled and GUS expression was investigated by X-gluc. The seedlings from which pieces of leaves were sampled were transplanted to 1/2N6 solid medium containing 50 mg/l hygromycin and cultured at 25°C for 10 days to check the hygromycin resistance. By this method, since resistant plants normally grow while sensitive plants die, resistance of the plants can be easily determined. Fragments of roots of some plants were transplanted on 2N6 solid medium (page 18, line 21-25 of the specification of the present application). Three weeks later, hygromycin resistance was determined depending on whether callus was formed from the root. By this method, since calli are formed from the roots of resistant plants while the roots of sensitive plants do not grow at all, resistance of the plants can be determined.

2. Southern Analysis of Introduced Genes

From the leaves of the 10 transformants used in 1 above, DNAs were extracted by the method of Komari et al. (Komari et al., 1989; Theoretical and Applied Genetics 77: 547-552), and the extracted DNAs were treated with a

restriction enzyme HindIII or KpnI. Using the HPT gene or GUS gene as a probe, detection of the introduced genes was carried out by Southern hybridization method. The extracted DNAs were also subjected to Southern hybridization method in which the DNAs were not treated with the restriction enzyme and in which the HPT gene was used as a probe. As controls, DNAs of a non-transformant and pTOK233 were used. The Southern hybridization method was carried out in accordance with Molecular Cloning (Sambrook et al., 1989; Cold Spring Harbor Laboratory Press). Eight self-pollinated progeny of one of the above-mentioned transformants (line number 6) were also subjected to the Southern analysis.

Results

3. Expression and Analysis of Introduced Genes in Primary Transformants and $R_1$ Progeny of Transformants

Total DNAs extracted from the 10 hygromycin-resistant and GUS-positive transformants were subjected to Southern analysis using the HPT gene as a probe so as to detect the introduced genes in the primary transformants. As a result, in all of the 10 transformants tested, the probe hybridized with high molecular weight DNAs in the nuclear genome of rice (Fig. 3). If *Agrobacterium* remains in the rice plant, the same signal as in the control plasmid pTOK233 (49.4 kb, Fig. 2) should be detected (Fig. 3). However, such a band was

not detected. This clearly confirms that the T-DNA was integrated into a rice chromosomal DNA.

Detection of the introduced genes in the primary transformants was carried out by subjecting the total DNAs extracted from the 10 hygromycin-resistant and GUS-positive transformants, which DNAs were digested with HindIII, to Southern analysis using the HPT gene as a probe. As a result, in all of the tested 10 transformants, one to several copies of the introduced genes were observed (Fig. 4). In the plasmid pTOK233, the HindIII fragment has a constant size of 5.1 kb (the arrows in Fig. 4 and Fig. 6). Therefore, if *Agrobacterium* remains in the rice plant, this band alone is detected. In this experiment, however, bands with varying lengths were detected in the tested transformants, respectively (Fig. 4). In addition, these bands were longer than the length (4.6 kb, Fig. 6) from the HindIII site to the left boarder via the GUS gene (Fig. 4). These results clearly prove that the T-DNA was integrated into different regions in chromosomal DNAs in the transformants.

Hygromycin resistance of the progeny of the transformants was checked. All of the seedlings of the non-transformants died in the presence of hygromycin (Table 15). In contrast, most of the seedlings of the progeny of the transformants normally grew in the presence of hygromycin (Table 15). All of the

hygromycin-resistant transformants exhibited expression of the GUS gene. In contrast, all of the hygromycin-sensitive plants did not exhibit GUS expression. Among the 10 lines tested, 6 lines showed genetic segregation in accordance with the one-factor segregation (3:1) for both the hygromycin resistance and expression of the GUS gene (Table 15). The remaining 4 lines showed genetic segregation in accordance with the two-factors segregation (15:1) for both the hygromycin resistance and expression of the GUS gene (Table 15). Table 15 also shows the results of the number of copies of the GUS gene in the primary transformants, which were obtained by Southern analysis. Although in some lines, the number of copies of the HPT gene and the GUS gene is different, this is thought to be due to the rearrangement of the introduced genes. This phenomenon is often observed in dicotyledonous transformants obtained by Agrobacterium-mediated transformation (Deroles and Gardner, 1988, Plant Mol. Biol. 11:365-377; Komari, 1989, Plant Sci., Theor. Appl. Genet., 80:167-171; Komari, 1990, Plant Cell Rep., 9:303-306).

Eight self-pollinated progeny of the transformant line number 6 were subjected to Southern analysis using the GUS gene as a probe. As a result, the GUS-negative and hygromycin-sensitive plants (lanes 7 and 8) did not have bands, while all of the GUS-positive and hygromycin-resistant plants (lanes 1-6) had the same single band as

the primary transformant. This line is one which was confirmed to have the one-factor segregation by the above-mentioned examination of the hygromycin resistance and the GUS expression, and the results of the Southern analysis proved the 1 copy of transgene and one-factor segregation.

These results clearly indicate that the genes introduced into rice by *Agrobacterium* were integrated into the genome of the rice cells and inherited according to Mendel's law. Further, these results clearly prove that the expression of the introduced genes by the rice plants was not due to the *Agrobacterium* possibly remaining in the rice tissues.

Table 15. Genetic analysis of independent transformants produced by LBA4404(pTOK233)

| Line No. of transgenic plans (R0)* | Copy number of Introduced genes (R0) | | Number of R1 plants | | $X^2$ values | |
|---|---|---|---|---|---|---|
| | *hpt* | *gus* | GUS-positive and hygromycin-resistant | GUS-negative and hygromycin-sensitive | 3:1 | 15:1 |
| C | - | - | 0 | 46 | - | - |
| 1 | 2 | 2 | 29 | 13 | 0.79 | 43.74** |
| 2 | 6 | 6 | 44 | 4 | 7.11** | 0.36 |
| 3 | 2 | 3 | 40 | 2 | 9.17** | 0.16 |
| 4 | 3 | 4 | 36 | 14 | 0.24 | 40.37** |
| 5 | 1 | 1 | 31 | 13 | 0.48 | 40.75** |
| 6 | 1 | 1 | 38 | 11 | 0.17 | 21.94** |
| 7 | 3 | 2 | 46 | 2 | 11.11** | 0.36 |
| 8 | 2 | 1 | 33 | 12 | 0.07 | 32.01** |
| 9 | 1 | 2 | 29 | 11 | 0.13 | 30.83** |
| 10 | 2 | 3 | 46 | 4 | 7.71** | 0.26 |

*C, non-transgenic plant ; **:significant at 1 % level.

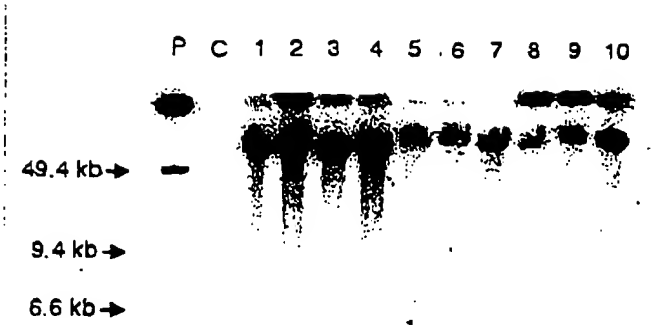


Figure 3. Southern analysis of primary transformants (undigested DNA).
DNAs extracted from pTOK233 (Lane P), a non-transgenic plant (Lane C) and 10 lines of transformants (Lane1-10) were fractionated by electrophoresis, transferred to nylon membrane, and allowed to be hybridized to the *hpt* probe. Line No. of tansformants was as the same as Lane No..

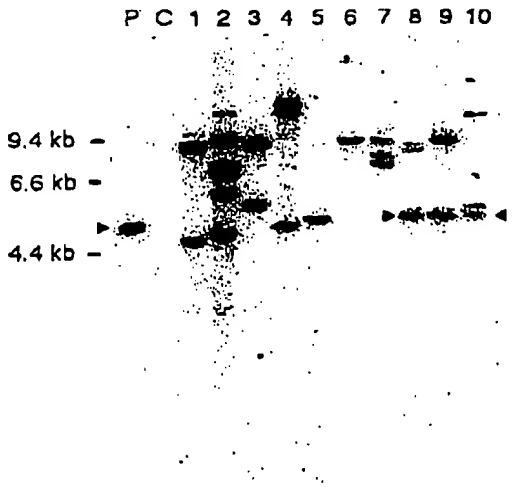


Figure 4. Southern analysis of primary transformants (digested DNA).
DNAs were as the same as Figure 3. Exception is that DNA of pTOK233 was added to three transformants DNA (Lane 8-10). These DNAs were digested with HindIII, fractionated by electrophoresis, transferred to nylon membrane, and allowed to be hybridized to the *hpt* probe.

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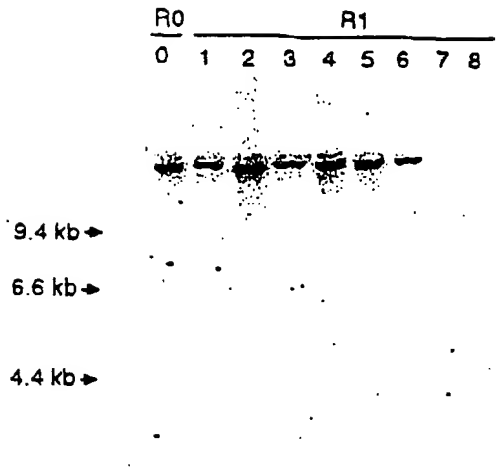


Figure 5. Southern analysis of the R1 progeny of transformant Line No. 6
DNAs extracted from R0 transformants Line No. 6 (Lane 0), hygromycin-resistant and GUS-positive R1 progeny (Lane 1-6), and hygromycin-sensitive and GUS-negative R1 progeny (Lane 7 and 8) were digested with KpnI, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to be hybridized to the *gus* probe.

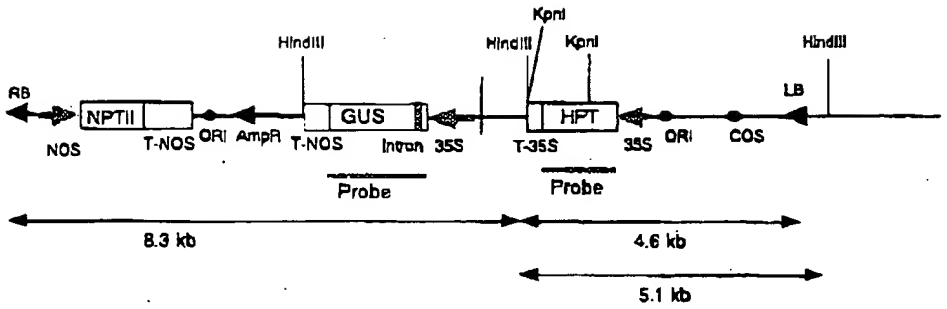


Figure 6. T-DNA region of pTOK233

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

This 9th day of November, 1998

Yukoh Hiei

Yukoh HIEI